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A 28-day rat inhalation study with an integrated molecular toxicology endpoint demonstrates reduced exposure effects for a prototypic modified risk tobacco product compared with conventional cigarettes

Ulrike Kogel [a,b,*], Walter K. Schlage [a,b], Florian Martin [b], Yang Xiang [b], Sam Ansari [a,b], Patrice Leroy [b], Patrick Vanscheeuwijck [a,b,c], Stephan Gebel [a,1], Ansgar Buettner [a,2], Christoph Wyss [b], Marco Esposito [b], Julia Hoeng [b], Manuel C. Peitsch [b]

[a] Philip Morris International R&D, Philip Morris Research Laboratories GmbH, Fuggerstrasse 3, 51149 Cologne, Germany
[b] Philip Morris International R&D, Philip Morris Products S.A., Quai Jeanrenaud 5, 2000 Neuchâtel, Switzerland
[c] Philip Morris International R&D, Philip Morris Research Laboratories bvba, Grauwmeer 14, Researchpark Haasrode, 3001 Leuven, Belgium

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ABSTRACT

Towards a systems toxicology-based risk assessment, we investigated molecular perturbations accompanying histopathological changes in a 28-day rat inhalation study combining transcriptomics with classical histopathology. We demonstrated reduced biological activity of a prototypic modified risk tobacco product (pMRTP) compared with the reference research cigarette 3R4F. Rats were exposed to filtered air or to three concentrations of mainstream smoke (MS) from 3R4F, or to a high concentration of MS from a pMRTP. Histopathology revealed concentration-dependent changes in response to 3R4F that were irritative stress-related in nasal and bronchial epithelium, and inflammation-related in the lung parenchyma. For pMRTP, significant changes were seen in the nasal epithelium only. Transcriptomics data were obtained from nasal and bronchial epithelium and lung parenchyma. Concentration-dependent gene expression changes were observed following 3R4F exposure, with much smaller changes for pMRTP. A computational-modeling approach based on causal models of tissue-specific biological networks identified cell stress, inflammation, proliferation, and senescence as the most perturbed molecular mechanisms. These perturbations correlated with histopathological observations. Only weak perturbations were observed for pMRTP. In conclusion, a correlative evaluation of classical histopathology together with gene expression-based computational network models may facilitate a systems toxicology-based risk assessment, as shown for a pMRTP.
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Abbreviations: AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care International; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; BIF, biological impact factor; CAM, cell adhesion molecule; COHb, carboxyhemoglobin; DEG, differentially expressed genes; EGAFS, ethanol glycerol acetic acid formaldehyde saline; FC, fold-change; FDA, Food and Drug Administration; FDR, false discovery rate; GC, gas chromatography; GCRMA, GC-Robust Microarray Analysis; GJIC, gap junction intercellular communication; GLP, Good Laboratory Practice; GSEA, gene set enrichment analysis; HCT, hematocrit; HPLC, high-performance liquid chromatography; KEGG, Kyoto Encyclopedia of Genes and Genomes; LCM, laser capture microdissection; LOQ, limit of quantification; MRTP, modified risk tobacco product; MS, mainstream smoke; NPA, network perturbation amplitude; OECD, Organization for Economic Co-operation and Development; pMRTP, prototypic modified risk tobacco product; RBIF, relative biological impact factor; RNE, respiratory nasal epithelium; TG, test guideline; TPM, total particulate matter; WHO, World Health Organization.

* Corresponding author at: Philip Morris International R&D, Philip Morris Products S.A., Quai Jeanrenaud 5, 2000 Neuchâtel, Switzerland. Tel.: +41 58 242 2600.
E-mail addresses: ulrike.kogel@pmi.com (U. Kogel), walter.schlage@contracted.pmi.com (W.K. Schlage), florian.martin@pmi.com (F. Martin), yang.xiang@pmi.com (Y. Xiang), sam.ansari@pmi.com (S. Ansari), patrice.leroy@pmi.com (P. Leroy), patrick.vanscheeuwijck@pmi.com (P. Vanscheeuwijck), stephan.gebel@uni.lu (S. Gebel), a.buettner@histovia.com (A. Buettner), christoph.wyss@pmi.com (C. Wyss), marco.esposito@pmi.com (M. Esposito), julia.hoeng@pmi.com (J. Hoeng), manuel.peitsch@pmi.com (M.C. Peitsch).
[1] Current address: Luxembourg Centre for Systems Biomedicine, 7, avenue des Hauts-Fourneaux, L-4362 Esch-sur-Alzette, Luxembourg.
[2] Current address: Histovia GmbH, Schöne Aussicht 5, 51491 Overath, Germany.

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1. Introduction

Historically, cigarettes and other tobacco products have been exempt from the health and safety standards governing contents and design that are typically applied to other consumer products including food and drugs (World Health Organization, 2007). "Unlike most products regulated by the Food and Drug Administration (FDA), tobacco is inherently hazardous and offers primarily risks rather than any significant physiological benefit to the user's health" (Institute of Medicine, 2012).

Recently, the Family Smoking Prevention and Tobacco Control Act of 2009 granted the FDA authority to regulate the manufacturing, distribution, and marketing of tobacco products, including "modified risk tobacco products" (MRTPs). An MRTP is defined by the Family Smoking Prevention and Tobacco Control Act as any tobacco product that is sold or distributed for use that reduces harm or the risk of tobacco-related disease associated with commercially marketed tobacco products (Food and Drug Administration, 2012a). MRTP applications must provide scientific evidence to demonstrate that the product significantly reduces harm and the risk of tobacco-related disease to individual users and benefits the health of the population as a whole, taking into account both users and non-users of tobacco products (Food and Drug Administration, 2012b). In this context, nonclinical studies play an integral role in the evaluation of MRTPs (Food and Drug Administration, 2012b).

The generation of tobacco smoke in a conventional lit-end cigarette is based upon an exothermic, self-sustaining combustion process generating some 6000 chemical compounds (Rodgman and Perfetti, 2013). Novel tobacco product designs have been developed that use distillation technology to heat rather than burn tobacco, thus lowering the extent of pyrolysis and quantity of combustion products (Coggins et al., 1989; Werley et al., 2008). We have previously reported on an electrically heated tobacco product (Schorp et al., 2012; Werley et al., 2008) but the prototypic MRTP (pMRTP) assessment reported here relates to a carbon heated tobacco product which contains a column of tobacco that is connected to a carbon heat source and is lit by the smoker via a standard lighter for use. The aerosol is created by gentle and controlled heating of the tobacco, which yields a smoke aerosol composed primarily of water and a humectant, for example glycerol, with reduced concentrations of combustion-related constituents such as aldehydes and polycyclic aromatic hydrocarbons.

Systems toxicology concepts such as the "21st Century Toxicology" (Mahadevan et al., 2011; Stephens et al., 2012) have recently been suggested to augment current toxicological risk assessment and to facilitate an understanding of the molecular mechanisms underlying the impact of biologically active substances/toxicants and associated disease risks.

With a view to developing a systems toxicology-based product risk assessment approach, the aims of this study were to: (a) demonstrate that the correlative evaluation of histopathology with gene expression analysis and advanced modeling tools is feasible within a classical Organization for Economic Co-operation and Development (OECD) inhalation study and can provide additional details about the molecular mechanisms underlying the test substance-related morphological changes, and (b) demonstrate reduced exposure effects for a single high dose of pMRTP compared with a conventional reference cigarette using this combined approach. To this end, the classical OECD TG 412 toxicological end points were combined with a transcriptomics analysis.

In this 28-day rat repeated dose inhalation toxicity study based on a modification of the Test Guideline 412 established by the OECD (OECD TG 412) (Organization for Economic Cooperation and Development, 2005), the biological activity of mainstream smoke (MS) from the pMRTP was determined and compared with that from the University of Kentucky Reference Cigarette 3R4F. OECD TG 412 studies are designed to characterize test article toxicity following repeated daily inhalation exposure, and assayed parameters were as specified in the OECD TG 412 (Organization for Economic Cooperation and Development, 2005) (in draft at the time the study was performed) and supplemented with pulmonary inflammation parameters from bronchoalveolar lavage (BAL). The required three dose levels were included for the reference cigarette, while only a single high dose of pMRTP was tested in this context to gain an initial understanding of the biological impact reduction potential of the pMRTP.

Within the same inhalation study, gene expression data were generated from the respiratory tract sites that were expected to be impacted by cigarette smoke (CS) and to show histopathological changes (Terpstra et al., 2003; Vanscheeuwijck et al., 2002). A novel computational-modeling approach based on our recently built tissue-specific networks (Gebel et al., 2013; Hoeng et al., 2012; Park et al., 2013; Schlage et al., 2011; Westra et al., 2011; Westra et al., 2013) was applied to place the molecular profiling data into the context of known biology. The mechanisms leading to perturbations in these networks were further analyzed with algorithms that quantify the amplitude of perturbed networks and provide an overall Biological Impact Factor (BIF) (Martin et al., 2012; Thomson et al., 2013).

2. Materials and methods

2.1. Experimental design

The study was designed and conducted according to OECD TG 412 with special emphasis on the histopathological evaluation of the respiratory tract. As an additional molecular end point to OECD TG 412, gene expression was investigated to further assess the exposure effects of MS from the 3R4F cigarette and potential differences between MS from the pMRTP and 3R4F. This analysis is therefore referred as the "OECD plus" part. Gene expression data were generated from tissue sites where histological changes are expected (Terpstra et al., 2003; Vanscheeuwijck et al., 2002) and from which sufficient tissue can be obtained for gene expression analysis, i.e., the respiratory nasal epithelium (RNE), lung bronchus, and lung parenchyma tissues. Thus, OECD TG 412 requirements were fulfilled for 3R4F, including the three dose levels tested, but we deviated from the guideline recommendation by testing only one high dose level of the pMRTP.

2.2. Cigarettes

Reference research cigarettes 3R4F were purchased from the University of Kentucky (http://www.ca.uky.edu/refcig/). pMRTP test articles were provided by Philip Morris Products S.A., Neuchâtel, Switzerland. The pMRTP is based on a design in which a carbon tip serves as a fast-lighting heat source for generating an aerosol containing water, glycerin, nicotine, and tobacco flavors, as well as reduced concentrations of tobacco pyrolysis products. 3R4F was selected as representative of a conventional cigarette and was compared with the pMRTP test article. Supplemental Table 1 shows the quantification of 51 harmful and potentially harmful constituents, water, and the humectant glycerol in 3R4F and pMRTP. Comparison of MS yields between pMRTP and 3R4F expressed and normalized to an equal nicotine basis showed that 33 constituents were reduced in pMRTP to less than 10% of 3R4F or to undetectable amounts (smoking was under Health Canada conditions (Health Canada, 1999)). Carbon monoxide (CO), acetaldehyde, acrolein, acetamide, 2-nitropropane, and catechol were reduced in pMRTP to 6–21% of that in 3R4F, whereas TPM increased to 131%, water to 277%, and glycerol to 507%. Thus, the yields of pMRTP were lower than 3R4F for all constituents except water and glycerol.

2.3. Animals

The study was conducted in an AAALAC-accredited facility (Association for Assessment and Accreditation of Laboratory Animal Care International, 2006) where the care and use of rats was conformed to the American Association for Laboratory Animal Science Policy (www.aalas.org). The study was approved by the local Institutional Animal Care and Use Committee according to Belgian legislation. It was conducted in compliance with the OECD Principles on Good Laboratory Practice (GLP) (as revised in 1997), with the exception of bronchoalveolar lavage fluid (BALF) analytics and transcriptomics investigations.

Outbred male and female Sprague–Dawley rats (Crl:CDBR), bred under specified pathogen-free conditions, were obtained from Charles River (L' Arbresle Cedex, France). Upon arrival, the health status was verified (by histopathological examina-

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